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## Research report

# Diazoxide and dimethyl sulphoxide prevent cerebral hypoperfusion-related learning dysfunction and brain damage after carotid artery occlusion

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## Abstract

Chronic cerebral hypoperfusion, a mild ischemic condition is associated with advancing age and severity of dementia; however, no unanimous therapy has been established to alleviate related neurological symptoms. We imposed a permanent, bilateral occlusion of the common carotid arteries of rats ( $n = 18$ ) to create cerebral hypoperfusion. A mitochondrial ATP-sensitive  $K^+$  channel opener diazoxide (DZ, 5 mg/kg) or its solvent dimethyl sulphoxide (DMSO) were administered i.p. (0.25 ml) on five consecutive days after surgery. Sham-operated animals ( $n = 18$ ) served as control for the surgery, while nontreated rats were used as control for the treatments. Three months after the onset of cerebral hypoperfusion, the rats were tested in a hippocampus-related learning paradigm, the Morris water maze. Subsequently, the animals were sacrificed and neurons, astrocytes and microglia were labeled with immunocytochemistry in the dorsal hippocampus. DMSO and diazoxide dissolved in DMSO restored cerebral hypoperfusion-related learning dysfunction and prevented cyclooxygenase-2-positive neuron loss in the dentate gyrus. Cerebral hypoperfusion led to reduced astrocyte proliferation, which was not clearly affected by the treatment. Microglia activation was considerably enhanced by cerebral hypoperfusion, which was completely prevented by diazoxide dissolved in DMSO, but not by DMSO alone. We conclude that diazoxide can moderate ischemia-related neuroinflammation by suppressing microglial activation. Furthermore, we suggest that DMSO is a neuroprotective chemical in ischemic conditions, and it must be considerably used as a solvent for water-insoluble compounds in experimental animal models.

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*Theme:* Disorders of the nervous system

*Topic:* Ischemia

*Keywords:* Cerebral hypoperfusion; Diazoxide; Dimethyl sulphoxide; Microglia; Spatial learning

## 1. Introduction

The incidence of chronic cerebral hypoperfusion increases with advancing age and dementia [15]; moreover, some researchers have found that decreasing cerebral blood flow (CBF) values correlate with an increasing degree of cognitive impairment in Alzheimer's disease patients [26,43]. In addition, reduced CBF has been recently suggested as an indicator for the progression of Alzheimer's disease [14,41]. Whether

reduced CBF is one of the triggers or the consequence of neuronal dysfunction cannot be conclusively decided, but experimental evidence suggests that decreased CBF can lead to cognitive impairment and neuronal injury [15].

The use of bilateral, permanent occlusion of the common carotid arteries of rats (2VO) is a well-characterized model to investigate the cognitive and histopathologic consequences of chronic cerebral hypoperfusion [15]. The hippocampus has been shown to be particularly affected because hippocampus-related spatial memory, neuronal and microvascular integrity were predominantly compromised in the region [8,15,52]. Several studies based on this model were designed

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to test potentially beneficial strategies to delay the progression of dementia associated with reduced CBF. For example, pharmacological treatment with cholinesterase inhibitors improved 2VO-related memory dysfunction, CBF, and cerebral metabolism [36,49]. Administration of plant extracts gained from *Ginkgo biloba* or huperzine-A also attenuated 2VO-induced learning deficit [30,60]. Alternatively, dietary supplements such as polyunsaturated fatty acids and antioxidants could moderate 2VO-imposed learning impairment or increase G-protein-coupled receptor densities in the hippocampus [11,16]. In our present study, we also made use of the 2VO model of cerebral hypoperfusion to test the potentially neuroprotective effect of diazoxide (DZ), a putative, mitochondrial ATP-sensitive potassium channel (mitoK<sub>ATP</sub>) opener.

Diazoxide has proved to be neuroprotective in several ischemia models. For instance, pretreatment with the agent could reduce infarct volume after middle cerebral artery occlusion in rats and mice [31,55]. Further in vivo experiments have provided evidence that diazoxide preserves NMDA-induced cortical arteriolar dilation after ischemia/reperfusion in piglets [12]. The mechanism behind these neuroprotective properties appears to be a selective opening of mitoK<sub>ATP</sub> because diazoxide effect can be abolished by the mitoK<sub>ATP</sub> blocker 5-hydroxydecanoate (5-HD) [24,32]. Hippocampal CA1 and CA3 interneurons are richly endowed by mitoK<sub>ATP</sub> that express region-specific distribution [18]. The activation of mitoK<sub>ATP</sub> in these cells under glucose deprivation proved to be neuroprotective as demonstrated in hippocampal slices [61]. Thus, the hippocampus appears to be a potential target and diazoxide a promising drug to achieve neuroprotection in chronic cerebral hypoperfusion.

Because diazoxide dissolves poorly in inorganic solvents, dimethyl sulphoxide (DMSO) was chosen in this study to prepare diazoxide solution. DMSO is widely used as a solvent for water-insoluble compounds in experimental animal research, yet DMSO itself possesses several vascular and neuroprotective properties [22,33,51]. For instance, DMSO can suppress platelet aggregation by antagonizing vasoactive substance release from platelets, or can scavenge neurotoxic free radicals [22,51]. Keeping these effects in mind, we also aimed to characterize and further explore the beneficial properties of DMSO in experimental cerebral hypoperfusion.

Our objective in the present study was to test the potentially neuroprotective effect of diazoxide in the 2VO model of cerebral hypoperfusion. Our prediction was that daily administration of the drug following carotid occlusion had beneficial effect on behavioral and neurological outcome. We were interested to investigate a late onset of complex changes caused by cerebral hypoperfusion.

In order to demonstrate drug effect in our experimental setup, we designed a Morris water maze paradigm frequently used as a standard test to assess hippocampus-related spatial memory function in 2VO [15]. Furthermore, we aimed to develop a comprehensive description of cerebral histopathologic changes by using neuronal, astroglial and microglial markers, of which the latest has not yet been employed in

experimental, chronic cerebral hypoperfusion. Cyclooxygenase-2 (COX-2) labeling was chosen to identify hippocampal neurons involved in ischemic injury because this inducible form of the enzyme is markedly expressed in transient ischemia [25,37,42]. Glial fibrillary acidic protein (GFAP) immunocytochemistry was employed to label astrocytes that can react by degeneration to reduced CBF in the hippocampus [57]. Finally, OX-42 antibody known to recognize CR3 complement receptors (CD11b) on microglia was used to identify microglial activation, which is an early event in ischemia [1,57].

## 2. Materials and methods

### 2.1. Surgery and treatment

Fifty-one male Wistar rats (210 ± 10 g) were used for the study. All animal experiments were approved by the ethical committee of the University of Szeged. Experimental cerebral hypoperfusion was imposed [16] on half of the animals by permanent bilateral occlusion of the common carotid arteries (2VO), the other half served as sham-operated controls (SHAM). Prior to surgery, the animals were anesthetized by 400 mg/kg chloral-hydrate i.p., followed by 0.05 ml atropine i.m. The common carotid arteries were exposed via a ventral cervical incision, and separated from their sheaths and vagal nerves. Silk sutures were used for the ligation. The same procedure was performed on the SHAM group without the actual ligation. Survival rates for each group are presented in Table 1.

Both groups were divided into three subgroups ( $n=6$ ) based on postsurgical treatment. The first set of animals of both SHAM and 2VO groups received 0.25 ml DMSO i.p., (SHAM–DMSO, 2VO–DMSO, respectively). The second set of animals was treated with 5 mg/kg diazoxide [7,31] given in 0.25 ml DMSO, i.p., (SHAM–DMSO + DZ, 2VO–DMSO + DZ). The animals were injected on five consecutive days. The first injection was applied directly after surgery. The last set of animals received no treatment after the operation and served as controls (SHAM–nontreated, 2VO–nontreated). The final composition of the experimental groups is presented in Table 1.

Table 1  
Survival rate and the incidence of CNS lesions

Experimental group		Survival rate (%)	Unilateral hippocampal lesion (%)	Unilateral cortical lesion (%)
Nontreated	SHAM	75 (6/8)	0.00 (0/6)	0.00 (0/6)
	2VO	66.6 (6/9)	66.67 (4/6)	33.34 (2/6)
DMSO	SHAM	100 (6/6)	0.00 (0/6)	0.00 (0/6)
	2VO	50 (6/12)	16.67 (1/6)	16.67 (1/6)
Diazoxide	SHAM	100 (8/8)	0.00 (0/6)	0.00 (0/6)
	2VO	87.5 (7/8)	0.00 (0/6)	0.00 (0/6)

Abbreviations: bilateral carotid artery occlusion (2VO), dimethyl sulphoxide (DMSO), sham operation (SHAM).

## 2.2. Spatial learning test

Eleven weeks after surgery, the animals were trained in the Morris water maze [11]. The water maze consisted of a polyester circular pool (diameter: 160 cm, height: 35 cm) filled with water (22 °C), which was made opaque by milk so that the rats were unable to see an underwater platform. The hidden platform was submerged 2 cm below water surface. The water tank was located in an experimental room with various extra maze cues to enable the rats to learn the location of the platform. All rats performed two trials per day with a constant intertrial interval of 4 h, for five consecutive days. The animals were placed in the water at one of four starting quadrant points, which was varied randomly over the trials. The rats were given 2 min to find the platform and sit on it for 15 s. Rats that failed to find the location within the given time were guided to the platform and were allowed to stay on it for 15 s. Swimming paths were recorded by a computerized video imaging analysis system (EthoVision, Noldus Information Technology BV, Wageningen, The Netherlands). At each trial, escape latency, and swimming distance traveled before reaching the platform were analyzed.

## 2.3. Immunocytochemistry

Ten days after the Morris water maze experiments, the animals were anaesthetized with an overdose of pentobarbital, and perfused transcardially with 100 ml saline followed by 400 ml 3.5% paraformaldehyde and 0.5% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed, one hemisphere was postfixed in the same solution for up to 1 h, and then stored in 0.1 M PB. Six brains per group were processed for immunocytochemical investigation.

Free floating coronal sections at the level of the hippocampus were cut at 20- $\mu$ m thickness on a cryostat. COX-2 labeling was performed on the first set of sections as follows. First, endogenous peroxidase activity was blocked with 0.3% H<sub>2</sub>O<sub>2</sub>. Nonspecific binding sites were covered with 5% normal goat serum (NGS) and membrane permeability was enhanced by 0.3% Triton X-100. The sections were incubated for 2 days at room temperature (RT) in primary antibody solution containing rabbit anti-COX-2 antibody (Cayman), 1:2000, 1% NGS, 0.3% Triton X-100 and 0.1% sodium azide in 0.01 M PBS (pH 7.4). Next, the sections were rinsed and preincubated in 5% NGS for 1 h. Incubation was performed in goat anti-rabbit biotinylated IgG (Santa Cruz) for 4 h at RT. Finally, the signal was amplified by an ABC–Avidin Kit (1:400) (Vector) for 2 h at RT. Color reaction was developed by diaminobenzidine (DAB) and H<sub>2</sub>O<sub>2</sub>.

A second set of sections was immunocytochemically stained for glial fibrillary acidic protein (GFAP) to visualize astrocytic proliferation. Briefly, sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS, and preincubated in 5% normal sheep

serum (NSHS). The samples were then incubated in a primary antibody solution containing mouse anti-GFAP antibody (Sigma), 1:200, 1% NSHS, and 0.3% Triton X-100 in 0.01 M PBS, overnight at 37 °C. The secondary antibody solution consisted of sheep anti-mouse biotinylated IgG (Jackson), 1:200, and 0.3% Triton X-100 in 0.01 M PBS. Finally, the sections were incubated in HRP–Streptavidine (Zymed), 1:200, and color reaction was conventionally developed with DAB and H<sub>2</sub>O<sub>2</sub>.

To detect and analyze microglial activation over the hippocampal areas, OX-42 antibody was used on a third set of sections. The procedure started with washing and pretreating the sections with 0.5% Triton X-100 and 3% H<sub>2</sub>O<sub>2</sub> in 0.01 M PBS, followed by preincubation in 20% normal swine serum (NSS) and 0.5% Triton X-100 in 0.01 M PBS for 1 h. The sections were incubated in a primary antibody solution containing biotinylated mouse anti-CD11b antibody (OX-42, Serotec), 1:500, 20% NSS, and 0.03% mertiolate in 0.01 M PBS, overnight at RT. Next, the sections were rinsed, and incubated in a solution of STA-PER (Jackson), 1% NSS, and 0.03% mertiolate in 0.1 M Tris-buffer for 1 h at RT. Finally, the color reaction was developed by Ni-DAB and H<sub>2</sub>O<sub>2</sub>. All sections were mounted on gelatin-coated microscopic slides, air-dried, dehydrated and coverslipped with DPX.

## 2.4. Analysis

Percentual surface area of GFAP-positive astrocytes was quantified in the dorsal hippocampus by using a Quantimet

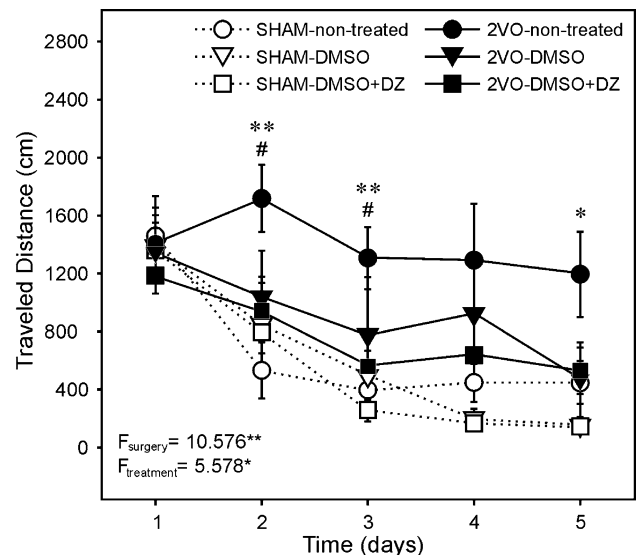


Fig. 1. Learning curves plotted of the Morris maze spatial orientation test. Statistic  $F$  values are based on a two-way repeated measurement ANOVA model ( $*p < 0.05$ ). Individual days were analyzed with ANOVA and LSD post hoc test ( $*p < 0.05$ ). (\*) indicates difference between SHAM–nontreated and 2VO–nontreated, (#) stands for the difference between 2VO–nontreated and 2VO–DMSO+DZ. Abbreviations: bilateral carotid artery occlusion (2VO), dimethyl sulphoxide (DMSO), diazoxide (DZ), sham operation (SHAM).



Q-600HR computerized image analysis system (Leica, Cambridge, UK) with a 469.4-nm emission filter [20]. Briefly, three consecutive coronal sections with a standard distance of 160  $\mu\text{m}$ , starting at Br. –3.60 mm [46] were selected for the analysis. Hippocampal regions of interest were manually delineated at  $10\times$  magnification, after background subtraction and gray scale threshold determination. The area covered by GFAP-positive astrocytes was computed as percentage of the total area delineated. Measurements of the three sections per animal were

averaged, and the average was used for further statistical analysis.

COX-2-labeled neuron counting was performed at  $20\times$  magnification with the help of an ocular mesh with 1600- $\mu\text{m}^2$  holes. Three consecutive coronal sections with a standard distance of 160  $\mu\text{m}$ , starting at Br. –3.60 mm [46] were examined. COX-2-positive neurons were counted in the CA3 stratum (str.) pyramidale and the dentate gyrus (DG) inner and outer str. moleculare on an average surface of 0.018  $\text{mm}^2$  in each region. Regional cell counts of three

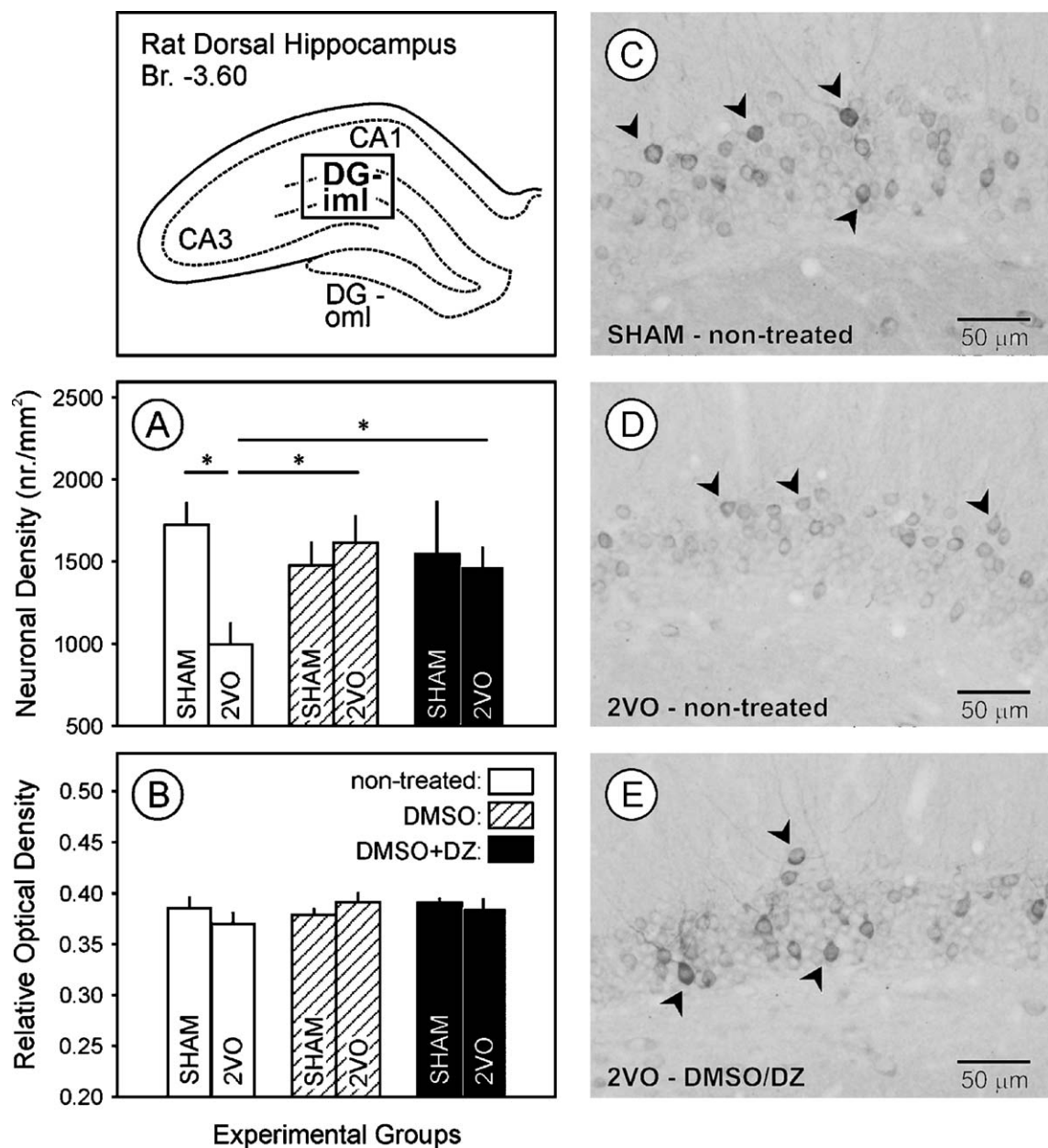


Fig. 2. Cyclooxygenase-2 enzyme (COX-2) immunocytochemistry in rat hippocampus. Panels A and B show neuronal density and COX-2 expression of preserved neurons, respectively. Significance values were obtained by Student *T*-test (\* $p < 0.05$ ). Panels C, D and E demonstrate COX-2-positive neurons in the dentate gyrus inner granular cells (arrowheads are pointing at a few labeled neurons). Abbreviations: bilateral carotid artery occlusion (2VO), dimethyl sulphoxide (DMSO), diazoxide (DZ), sham operation (SHAM).

sections per animal were averaged, and the average was used for further statistical analysis.

The optical density of COX-2-positive neurons was measured by using a Quantimet Q-600HR computerized image analysis system (Leica). The same sections used for COX-2-labeled cell counting were analyzed again, at  $10\times$  magnification. Following background subtraction and gray scale threshold determination, COX-2-positive neurons were automatically delineated by the program, and relative optical density was computed based on a standard gray scale. As done previously, values of three sections per animal were averaged, and this value was used for further statistical analysis.

Quantification of the surface covered by OX-42 immunoreactive microglia was performed in a similar manner to GFAP-labeled sections on a computerized image analysis system (Olympus BX50, DP50; software: ImagePro Plus, Media Cybernetics).

The Morris maze test results were statistically analyzed by a two-way repeated measurement model followed by LSD post hoc test of the program SPSS. Individual day comparisons were performed by a univariate model and LSD post hoc analysis of SPSS. GFAP and OX-42 immu-

nocytochemical results were statistically analyzed with two-way ANOVA followed by LSD post hoc test.

### 3. Results

Spatial learning curves obtained in the Morris maze test (Fig. 1) demonstrated that experimental cerebral hypoperfusion induced a marked decrease in learning performance ( $**p < 0.002$ ). Moreover, hardly any day-to-day improvement was observed in the nontreated 2VO group over the 5-day training period. Daily comparison of groups revealed that the nontreated 2VO animals' memory capacity was significantly worse than their respective SHAM controls specifically on days 2, 3, and 5. Conversely, either DMSO or DMSO+DZ treatment improved learning of 2VO rats compared to the nontreated 2VO group, ( $*p < 0.040$  and  $**p < 0.007$ , respectively). On the other hand, the agents did not affect the performance of SHAM animals. Furthermore, statistical analysis of the daily performance showed significant improvement only in the DMSO-DZ treated 2VO rats compared to the nontreated 2VO group on days 2, and 3.

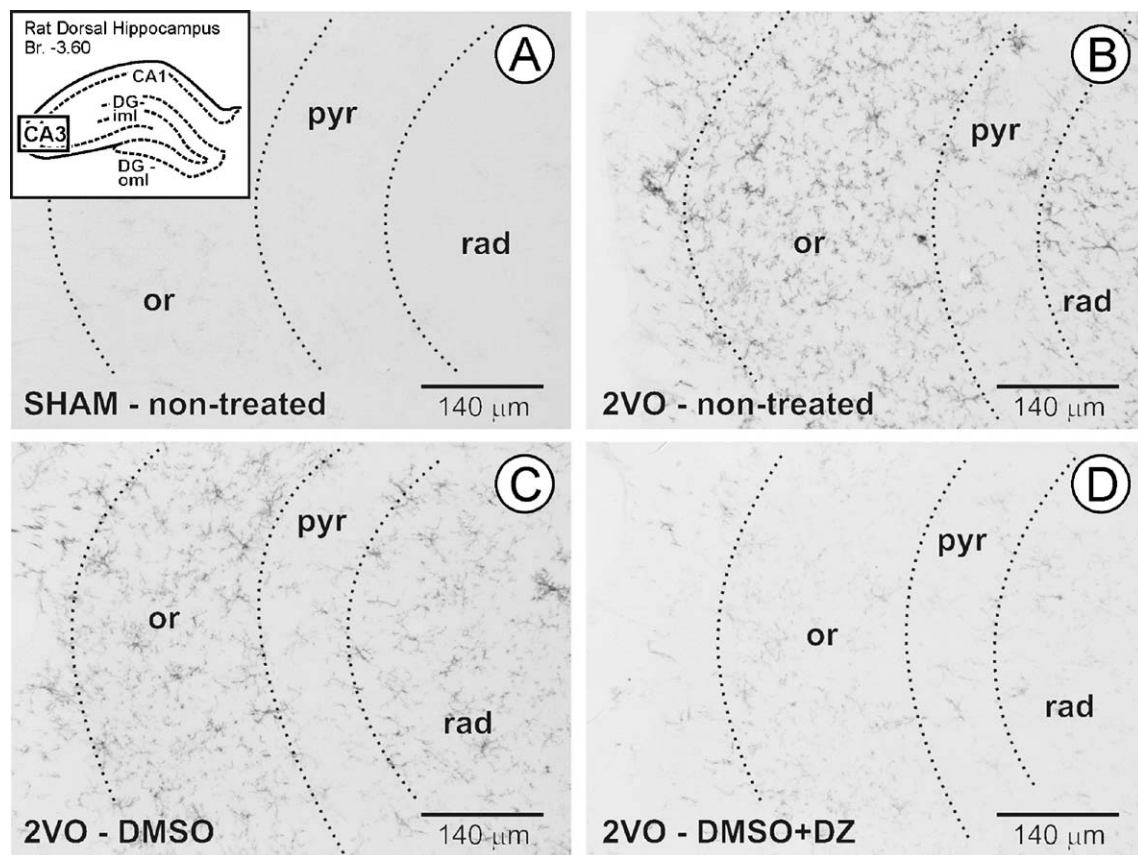


Fig. 3. Microglial activation in the rat hippocampus CA3 region labeled with OX-42 immunocytochemistry. A: image from a SHAM non-treated animal. B: image from a 2VO non-treated animal. C: image from a 2VO, DMSO-treated animal. D: image from a 2VO, DMSO+DZ-treated animal. Images were taken at  $10\times$  magnification. Abbreviations: 2VO: bilateral carotid artery occlusion, DMSO: dimethyl sulphoxide, DZ: diazoxide, or: str. oriens, pyr: str. pyramidale, rad: str. radiatum, SHAM: sham-operation.

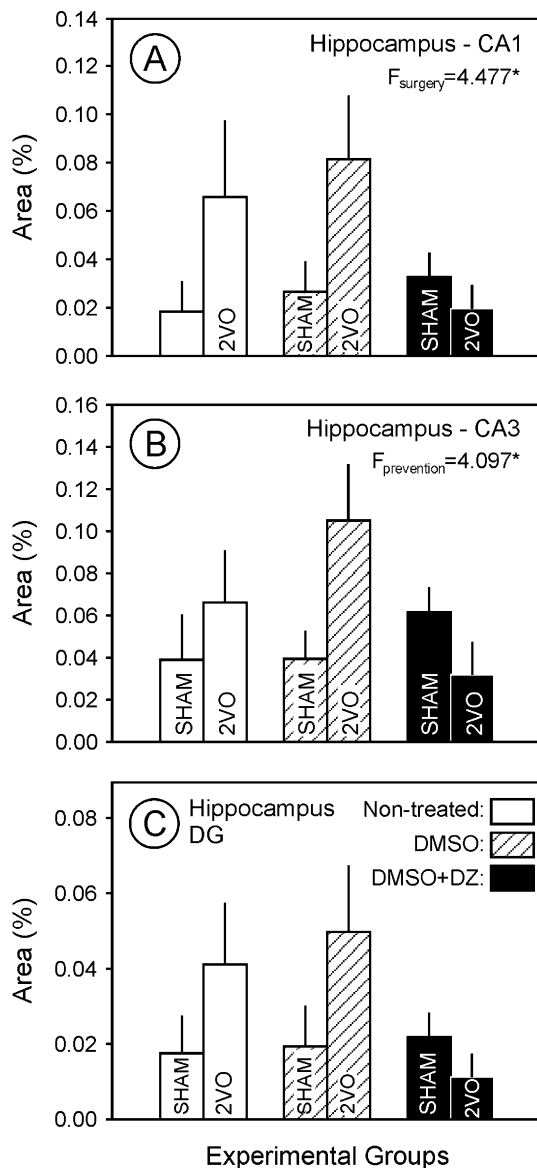


Fig. 4. Quantitative analysis of OX-42-positive microglial activation. A: hippocampus CA1 region. B: hippocampus CA3 region. C: hippocampus dentate gyrus. Statistic  $F$  values are based on a two-way ANOVA model ( $*p < 0.05$ ). Abbreviations: 2VO: bilateral carotid artery occlusion, DMSO: dimethyl sulphoxide, DZ: diazoxide, SHAM: sham-operation.

Macroscopic evaluation of rat brains demonstrated that 2VO without postoperative treatment induced hippocampal and cortical lesions (in 66.67% and 33.34% of the animals, respectively). DMSO application reduced, while DMSO + DZ completely abolished the occurrence of the lesions (Table 1).

COX-2 immunocytochemistry in the dorsal hippocampus labeled neurons in the stratum pyramidale, the hilus and granular cells of the inner stratum moleculare of the dentate gyrus (DG-iml) (Fig. 2). The outer stratum moleculare of the DG contained only very few, scattered labeled neurons. Cell counting revealed loss of about 50% of COX-2-positive neurons in the nontreated 2VO group specifically in the

DG-iml. Both DMSO and DMSO + DZ treatments prevented neuronal loss in the region (Fig. 2A). Optical density measurements showed that the surviving cells expressed an unaltered amount of COX-2 (Fig. 2B).

Glial fibrillary acidic protein (GFAP) immunoreactive astrocytes were present in all hippocampal regions and in all experimental groups. The area covered by astrocytes showed a similar tendency to COX-2-positive neuron counting in that astrocytic proliferation was reduced in 2VO compared to SHAM. However, no clear effect of the treatments could be established (data not shown).

OX-42 positive activated microglia were scarce in the hippocampus of SHAM animals (Fig. 3A), while a dense staining could be observed in all hippocampal areas in the nontreated and DMSO-treated 2VO groups (Fig. 3B and C). On the other hand, DMSO + DZ but not DMSO alone diminished OX-42-positive microglia in 2VO rats (Fig. 3D). Quantitative analysis demonstrated that 2VO increased microglia activation from two to three times in the CA1, CA3 and DG molecular layers in the nontreated and DMSO-treated 2VO animals. The increase was statistically significant in the CA1 region ( $*F = 4.477$ ). Application of DMSO + DZ but not DMSO alone reduced microglia activation to SHAM control level in all investigated regions, but the preventive effect of DMSO + DZ was statistically significant only in the CA3 area ( $*F = 4.097$ ) (Fig. 4).

#### 4. Discussion

The novel findings of this study are the following. Chronic administration of DMSO following 2VO can moderate ischemia-related memory failure, and diazoxide in concert with DMSO can virtually prevent memory dysfunction. In a similar way, DMSO can confine, and diazoxide applied in DMSO can completely diminish macroscopic hippocampal and cortical lesions. Furthermore, DMSO alone is sufficient to prevent COX-2-positive neuron loss in the DG. Finally, diazoxide, but not DMSO alone can attenuate microglia activation in the hippocampus.

Cerebral hypoperfusion created by 2VO has been repeatedly described to cause spatial memory dysfunction [8,11,15,39,45], and apoptotic neuronal death in the hippocampus of rats [6,19,40]. Similarly, our Morris maze experiment reproduced earlier observations that chronic 2VO causes spatial memory impairment [8,15,39,45]. On the other hand, DMSO, and particularly DMSO + DZ restored learning skills to nearly SHAM level. To the best of our knowledge, the effects of diazoxide on learning and memory have not yet been tested. In addition, only one prior study attempted to identify the potentially beneficial effect of DMSO on spatial learning skills, but in that study DMSO was not administered alone but was combined with fructose 1,6-diphosphate [10].

In addition, our examination of rat brains following 3 months 2VO revealed macroscopic hippocampal and corti-



cal lesions in four out of six untreated animals. The lesions can very well arise from acute ischemic strokes as reported by early studies on the 2VO model, and also correlate with the regional distribution (hippocampus and neocortex), and ratio of affected animals (35–65%) in previous experiments [21,34,50]. Chronic treatment with DMSO reduced, while DMSO+DZ abolished, such ischemic lesions. Because DMSO can improve hemodynamic variables and CBF [23,28,59] in addition to its known free radical scavenger properties [2,51], an increased flow or reduced concentration of free radicals may underlie the preventive effect of the agent. Our observation also supports the data that DMSO could reduce infarct volume in focal cerebral ischemia [47,54]. Diazoxide was also found to be neuroprotective in ischemia–reperfusion brain injury [55]. Therefore, the cumulative effect of DMSO and diazoxide could be responsible for the complete prevention of lesions. Furthermore, such a protective action accomplished by the drugs corresponds with the treatment-related improvement in spatial learning skills demonstrated in our study.

The distribution and temporal aspects of COX-2 expression have been well characterized in the hippocampus after transient but not permanent forebrain ischemia [25,37,44]. The enzyme was suggested to promote neuronal death because selective COX-2 inhibitors could prevent ischemic injury in the hippocampus and other brain areas [13,37,42]. In our permanent cerebral hypoperfusion model, the pattern of COX-2-positive neurons in the hippocampal CA3 and DG appeared to be very similar to that seen with *in situ* hybridization 3–4 days postischemia, following 5–20 min global ischemia–reperfusion [25,37]. However, the labeling was also present in SHAM animals in our studies as well as other studies [37]. Thus, the data here strongly support the assumption that the acute onset of ischemia rather than chronic cerebral hypoperfusion or neuroinflammation is the condition that induce neuronal COX-2 expression. Furthermore, we detected a drop in COX-2-positive neuron density in the DG inner granular cells in untreated 2VO rats, which was not accompanied by altered COX-2 expression in surviving cells. Because COX-2 had been expected to be upregulated in ischemia, the lower number of labeled neurons compared to SHAM control might indicate neuronal death in the COX-2 producing cell population. Both DMSO and DMSO+DZ treatments prevented neuronal loss to an equal degree, which suggests that DMSO administration was sufficient to preserve neuronal integrity in the DG.

We found reduced astrocytic proliferation in 2VO rats, which corresponds with a previously described dynamics of astrocytic reaction in the 2VO model. In transient 2VO, no change was detected in astrocyte number up to 2 weeks in case of a 5-min occlusion, but a gradual degeneration and loss of GFAP-positive astrocytes were seen with the longer duration of the ischemic period or survival time [57]. Because our samples were obtained 3 months after the onset of permanent 2VO, the decline of GFAP signal could be due to a similar, progressive loss of astrocytes. Such degeneration

of astrocytes can have serious functional consequences because an interaction between astrocytes, neurons and the cerebral microcirculation is essential to maintain neural energy metabolism and synaptic plasticity [3–5].

Finally, microglial activation was remarkably augmented by 2VO, which could be completely prevented only with DMSO+DZ, and not with DMSO alone. Microglia activation is an early response in the neuroinflammatory reaction to ischemia and was repeatedly detected with OX-42 immunocytochemistry in the hippocampus during the acute phase of ischemia [1,38,57]. However, there is no clear data on microglial reaction in chronic cerebral hypoperfusion models. Our results here show that microglial activation persists over a long period of time after the onset of chronic ischemia, which may suggest an ongoing neuroinflammatory process that accompanies chronic cerebral hypoperfusion.

Microglia may serve as part of regenerative processes by scavenging necrotic tissue, but can also promote delayed neuronal damage by generating cytotoxic agents, such as proinflammatory cytokines [27,56]. Consequently, the lower activation state of microglia due to DMSO+DZ in our study may be interpreted in two alternative ways. The treatment may have confined neuronal damage that would recruit microglia to clean up necrotic debris. On the other hand, microglia activation itself could have been directly inhibited, which would implicate delayed neuroprotection via the restricted production of cytotoxic compounds. We believe the latter is more probable because neuronal damage that recruits microglia is an early event in stroke, and our samples were obtained 3 months after the onset of ischemia.

Because DMSO+DZ limited microglia activation but DMSO alone did not, this effect can be attributed specifically to diazoxide. The beneficial action of diazoxide on neurons and astrocytes has been studied in detail [12,31,48], but no such data is available on microglia. Therefore, these findings may trigger further experiments to identify through which pathways diazoxide can alter microglial activation, and how exactly it may contribute to the outcome of ischemic insults.

Based on our data, we have no direct evidence as to the exact mechanism of DMSO and diazoxide that was responsible for the improvement of behavior and neuronal integrity. Yet, we speculate that the two agents achieved neuroprotection through different, but to some extent complementary pathways. DMSO has long been proposed to be protective against ischemia by antagonizing platelet aggregation and platelet-related vasoconstriction, or by neutralizing hydroxyl radicals [9,22]. Conversely, diazoxide has recently come to the focus for mimicking ischemic preconditioning by selectively opening mitochondrial  $K_{ATP}$  channels [58]. Therefore, the target of DMSO could be the cerebral microcirculation, while diazoxide probably acted directly on neural compartments.

In spite of its known pharmacological actions, DMSO is frequently used as a solvent for diazoxide [17,29]. Based on



our results, and because DMSO itself is a potent neuroprotective chemical, an alternative method (e.g., NaOH and saline) is recommended to dissolve diazoxide in future studies designed for the investigation of neuroprotection through  $K_{ATP}$  channel opening.

Finally, diazoxide in this study was used unconventionally as a posttraumatic agent. Diazoxide is usually applied as pretreatment before CNS insults because the drug is known to mimic the effects of ischemic preconditioning [12,31,35,53]. Our data that diazoxide (dissolved in DMSO) can be potentially neuroprotective given in a posttraumatic manner may open up new possibilities as to the therapeutic application of the drug.

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